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Status report covers progress made through the period of February 1, 1990 - October 31, 1990 on N00014-89-J-1916, PI: Judy A. Spitzer, Ph.D.

STATUS REPORT
N00014-89-J-1916
FOR FEBRUARY 1, 1990 - OCTOBER 31, 1990

Progress was made in the following areas:

1. <u>Evaluation of host defense mechanisms by measurement of superoxide anion release in alveolar macrophages.</u>

   A. <u>Alveolar macrophages from TNF-treated rats</u>

TNF (recombinant human TNF from Cetus Immune Corp.) was administered in three different modes, a 3 hour infusion, an intravenous bolus injection into the penile vein with sacrifice at 90 min, and intratracheal TNF bolus injection with sacrifice at 90 min.
Spontaneous and opsonized zymosan (OZ)-stimulated release of superoxide anion (SO) was measured in alveolar macrophages (AMs) from rats after a 3 hour infusion of 6 x 10^5 U of TNF or saline. Since this experimental design did not lead to consistent differences between the experimental and control groups studied, we elected to use the bolus injection model with sacrifice time at 90 min after the i.v. injection of TNF (6 x 10^5 units in a total volume of 0.5ml.) In this protocol, both OPZ and the tumor promoter phorbol ester, PMA were used in the assessment of SO generation. The zymosan dose response studies revealed no consistent differences between the AMs obtained from TNF- or saline-treated rats. However, TNF treatment resulted 90 min later in a statistically significant increase in PMA-stimulated SO production. See Figure 1.

Ths consequences of a TNF bolus injection via the intratracheal route (6 x 10^5 units in 0.5 ml total volume) were also examined. Spontaneous and agonist-stimulated release of SO was measured in AMs lavaged from rats 90 min after an intratracheal bolus injection of TNF. The agonists used, PMA (10^{-6}M), OZ (312 μg/ml) and A23187 (10^{-6}M) were selected because of their diverse physical characteristics i.e. soluble versus particulate, and taking into account that the effects of these agonists are mediated through different transduction mechanisms. With the intratracheal model, just as with the i.v. bolus injection, PMA stimulation of AMs leads to consistently increased SO release as compared to AMs of saline-treated animals. A PMA dose response curve obtained with AMs after intratracheal administration of TNF is presented in Figure 2. The response to OZ stimulation in such AMs is marked, but there are no consistent differences between the two treatment groups. Likewise, the response to the calcium ionophore, A23187, does not appear to have a consistent difference and the response is rather weak.

B. Some mediating mechanisms in opsonized zymosan (OZ) -stimulated superoxide anion (SO) release in alveolar macrophages obtained from endotoxin- (ET) treated rats.

This work was a continuation of the studies that we reported on in the previous status report. Various signal transduction mechanisms were explored in the bolus injection model (90 min sacrifice time). Phospholipid turnover data obtained in AMs labeled with $(^{32}P)P_i$ suggested that OZ stimulation results in increased accumulation of ^{32}P-PA, probably by phospholipase C- (PLC) mediated stimulation of the PI cycle, but also possibly by phospholipase D- mediated hydrolysis of phosphatidylcholine. OZ stimulation had no effect on the turnover of ^{32}P-PE, but did result in decreased ^{32}P-PC and increased ^{32}P-LPC (lysoPC), suggesting phospholipase A_2 (PLA_2)-mediated hydrolysis of PC).
Experiments were subsequently designed to explore the influence of a PLA_2 inhibitor, mepacrine, on OZ-stimulated SO release. The results of these studies demonstrate that PLA_2-mediated pathways are essential for SO release in both saline- and ET-treated animals (Figure 3). ET did not effect OZ-stimulated release of ^{32}P-LPC, but OZ stimulation resulted in a lower accumulation of ^{32}P-PA in AMs from the ET-treated group. This indicates that ET does not affect the ability of OZ to stimulate PLA_2, but ET may suppress OZ stimulation of the PI cycle.

The exact manner in which stimulation of the PI cycle is coupled to SO release is not understood at this time. One of the possible ways in which PI cycle stimulation may be coupled to NADPH oxidase activation and SO release involves activation of protein kinase C (PKC) by DAG, one of the messengers produced during stimulation of the PI cycle and the fact that activation of PKC by phorbol esters can stimulate the respiratory burst. Data obtained in our studies indicate that PKC plays a role in SO release in OZ-stimulated rat AMs. In AMs from both saline controls and ET-treated rats, inhibition of PKC by staurosporine suppressed SO release in a dose-dependent fashion (Figure 4).

Fig. 5. depicts the relationship of OZ-stimulated signal transduction pathways (involving PLC-mediated stimulation of the PI cycle, PLA_2-mediated hydrolysis of PC, and PKC activation) to NADPH oxidase activation and O_2^- (SO) release.

The significance of these data is constituted by the finding that after 90 min of an i.v. bolus injection of TNF, AMs are primed to respond to a soluble stimulus, namely PMA by a significantly increased SO release compared to corresponding cells lavaged from saline-treated control animals. It is of further interest to note that as shown by other investigators, previously injected TNF is compartmentalized, i.e. TNF injected into the vascular compartment cannot be demonstrated to reach the alveolar compartment (Nelson, S., Bagby, G.J., Bainton, B.G., Wilson, L.A., Summer, W.R. J.Infect.Dis. 159:189-194, 1989). Nevertheless, the priming effect of the injected cytokine can be demonstrated in AMs. Enhanced host defense mechanisms in terms of increased SO release can also be

observed in AMs lavaged after 90 min of an intratracheal administration of TNF. This mode of TNF administration results in the priming of AMs in response to PMA stimulation, but it does not extend to an increased SO release in response to OZ or A23187 stimulation.

The data obtained with i.v. ET administration demonstrate alterations in phospholipid turnover of PLC- and PLA_2-mediated pathways. PLA_2- and PKC-mediated events are implicated in the OZ release of SO in AMs from both control and ET-treated animals. ET produces a transient enhanced OZ-stimulated SO release in AMs as determined in vitro. This may be the consequence of priming via a soluble mediator capable of cross compartmental communication and we propose platelet activating factor (PAF) as being the likely mediator.

2. Superoxide anion release in Kupffer cells

Spontaneous and agonist (PMA 10^{-6}M, OZ 312 ug/ml and A23187 1μM)-stimulated release of SO was also measured in Kupffer and endothelial cells isolated from rats at the end of a 30 hr continuous ET-infusion. As shown in Figures 6-8, Kupffer cells produced significantly more SO upon PMA and OZ stimulation than did Kupffer cells from saline infused animals. Endothelial cells produced very little SO upon stimulation by any of the three agonists used.

The significance of these data lies in the fact that they demonstrate in vivo priming of Kupffer cells due to a continuous infusion of a nonlethal dose of ET that is manifested when the cells are challenged in vitro by either a particulate or a soluble stimulus. The data also suggest that the enhanced respiratory burst and therefore the enhanced host defense mechanism exhibited by these cells is likely to be mediated by PKC related mechanisms, whereas pathways involving calcium fluxes do not appear to be contributing to this response.

I was going to present some of our results related to SO release and signal transduction in rat alveolar macrophages at the 12th International RES Congress that was held October 14-18, 1990 in Crete, Greece. Due to the rather uncertain situation in the Middle East, I canceled this trip to the Mediterranean region. I am planning to present these results in the future at an appropriate meeting.

Issues that may affect our research progress: At this time, we have only positive things to report. Dr. Alejandro M. S. Mayer finally joined my laboratory on May 1, 1990, and has contributed significantly to the progress reported here. In addition, an experienced Research Associate, Ms. Carol Hoppens, was hired September 1, 1990. She is a very competent person who has learned the techniques involving the preparation of AMs and all the biochemical techniques that we use in our research related to these cells. Ms. Betty Dowty, my Ph.D. student, whose dissertation "Effect of in vivo administration of endotoxin on signal transduction mechanisms for superoxide anion release by rat alveolar macrophages" is based on work supported by this grant, has completed her graduate work and is going to stay with us until next August as a postdoctoral fellow. She will continue the line of research that she has been successful with so far.

List of abbreviations used:

AM - alveolar macrophage
OZ - opsonized zymosan
PMA - phorbol myristate acetate
SO - superoxide anion
PA - phosphatidic acid
PI - phosphatidyl inositol
PC - phosphatidyl choline
PE - phosphatidyl ethanolamine
LPC - lysophosphatidyl choline
PLA_2 - phospholipase A_2
PLC - phospholipase C
PKC - protein kinase C
DAG - diacylglycerol
ET - endotoxin
TNF - tumor necrosis factor

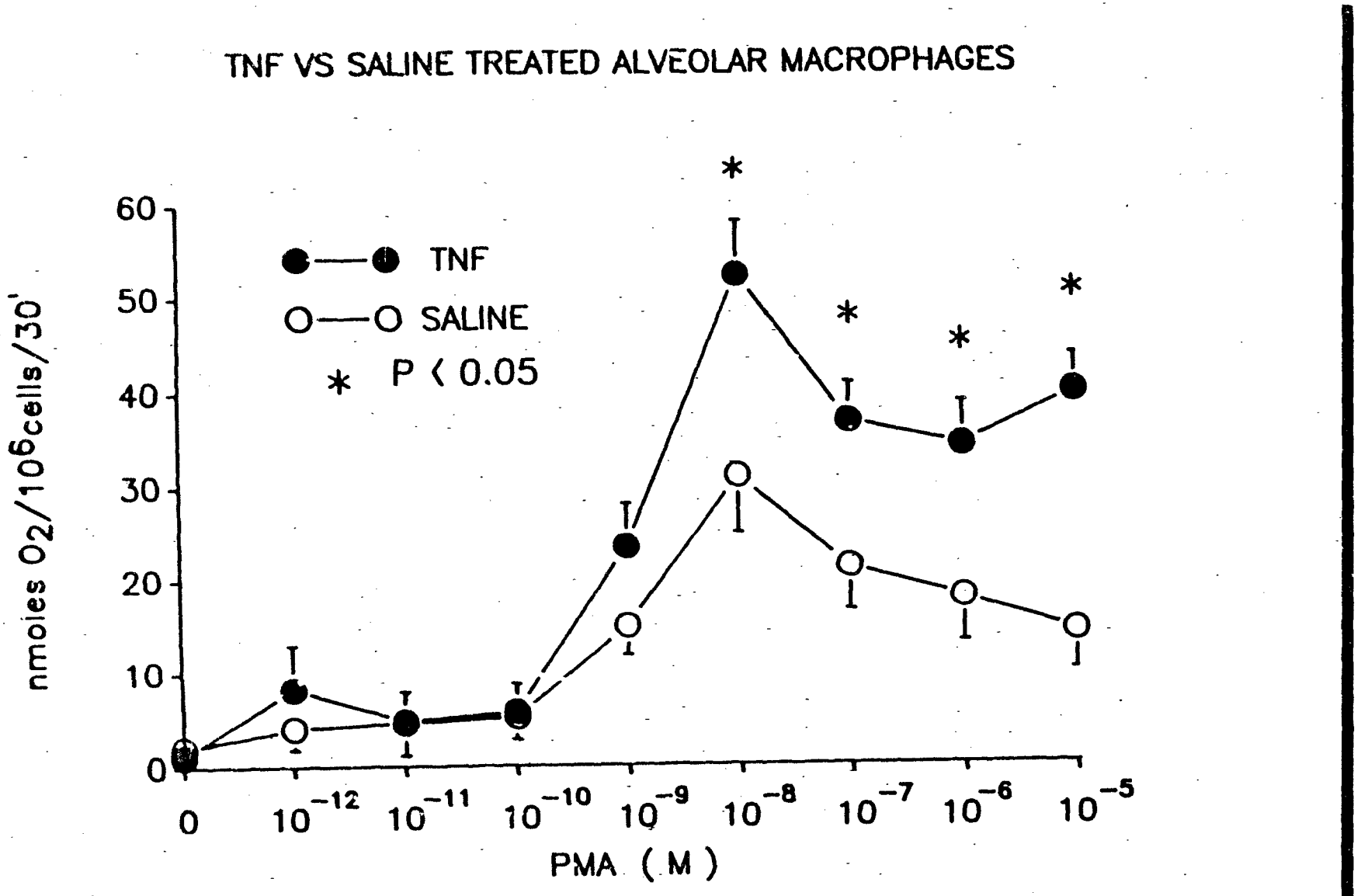

Fig. 1. PMA-dose response of SO release by alveolar macrophages of TNF- and saline-injected rats. (i.v. bolus injection, n = 4)

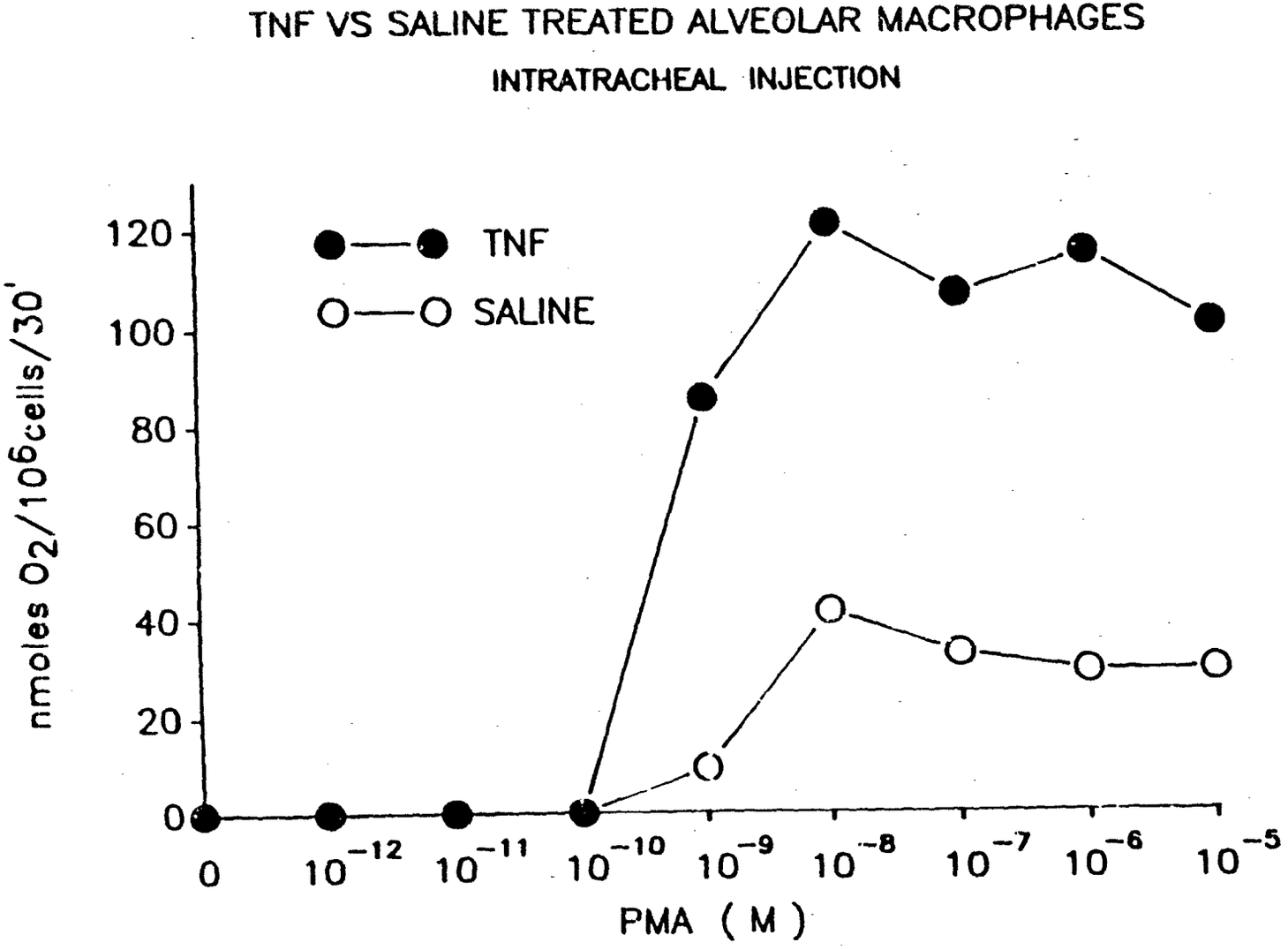

Fig. 2. PMA-dose response of SO release by alveolar macrophages of a TNF- and a saline-injected rat (intratracheal injection).

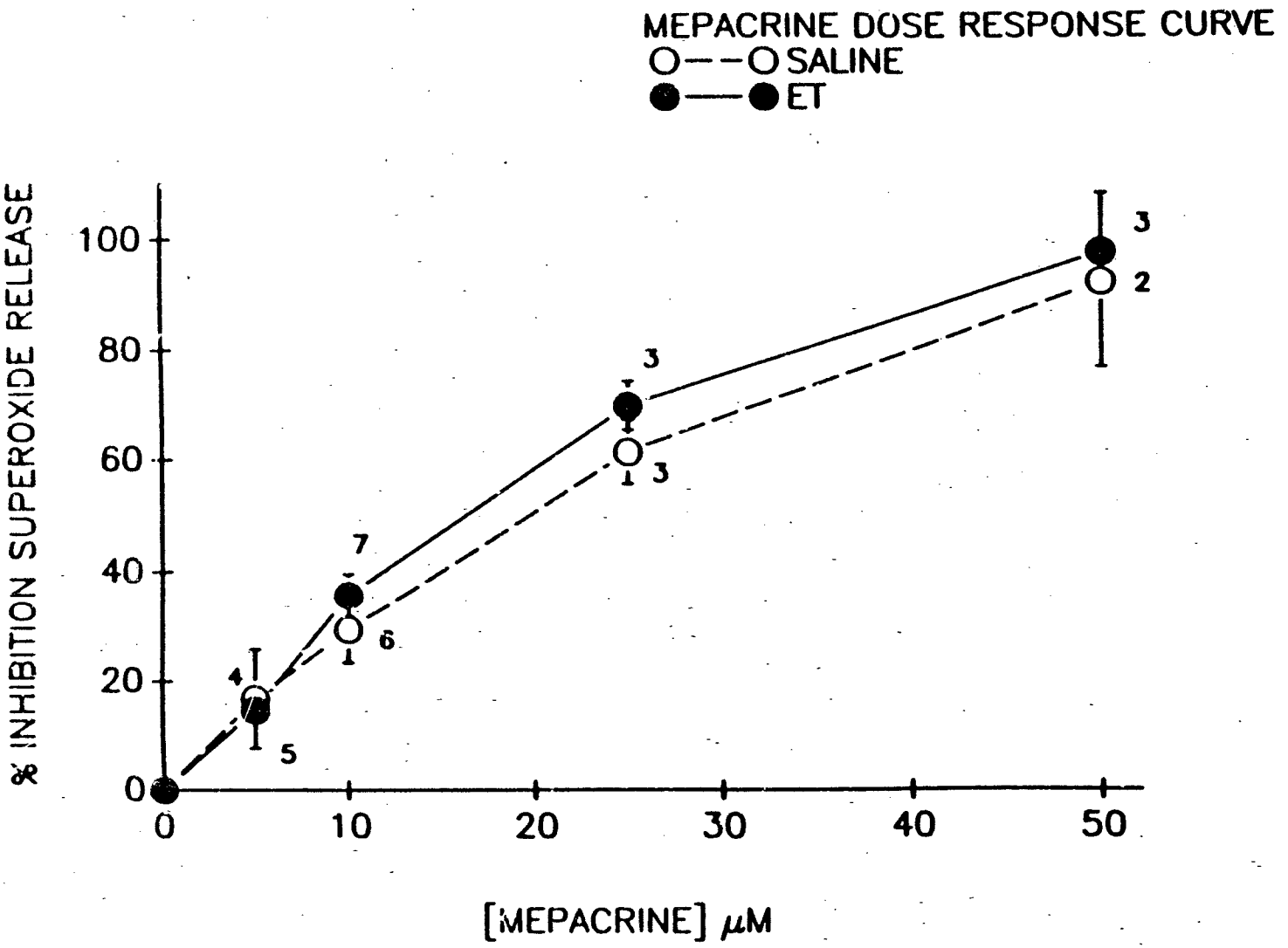

Fig. 3. Effect of mepacrine on SO release in AMs stimulated with OPZ (3.4 mg/kg b.w.). AMs were pre-incubated with the indicated concentration of mepacrine for 15 minutes at 37°C in a shaking water bath prior to the SO assay. AMs were isolated from male Sprague-Dawley rats 1.5 hours following an i.v. bolus injection of either saline or a nonlethal dose of ET (500 ug/kg b.w.).

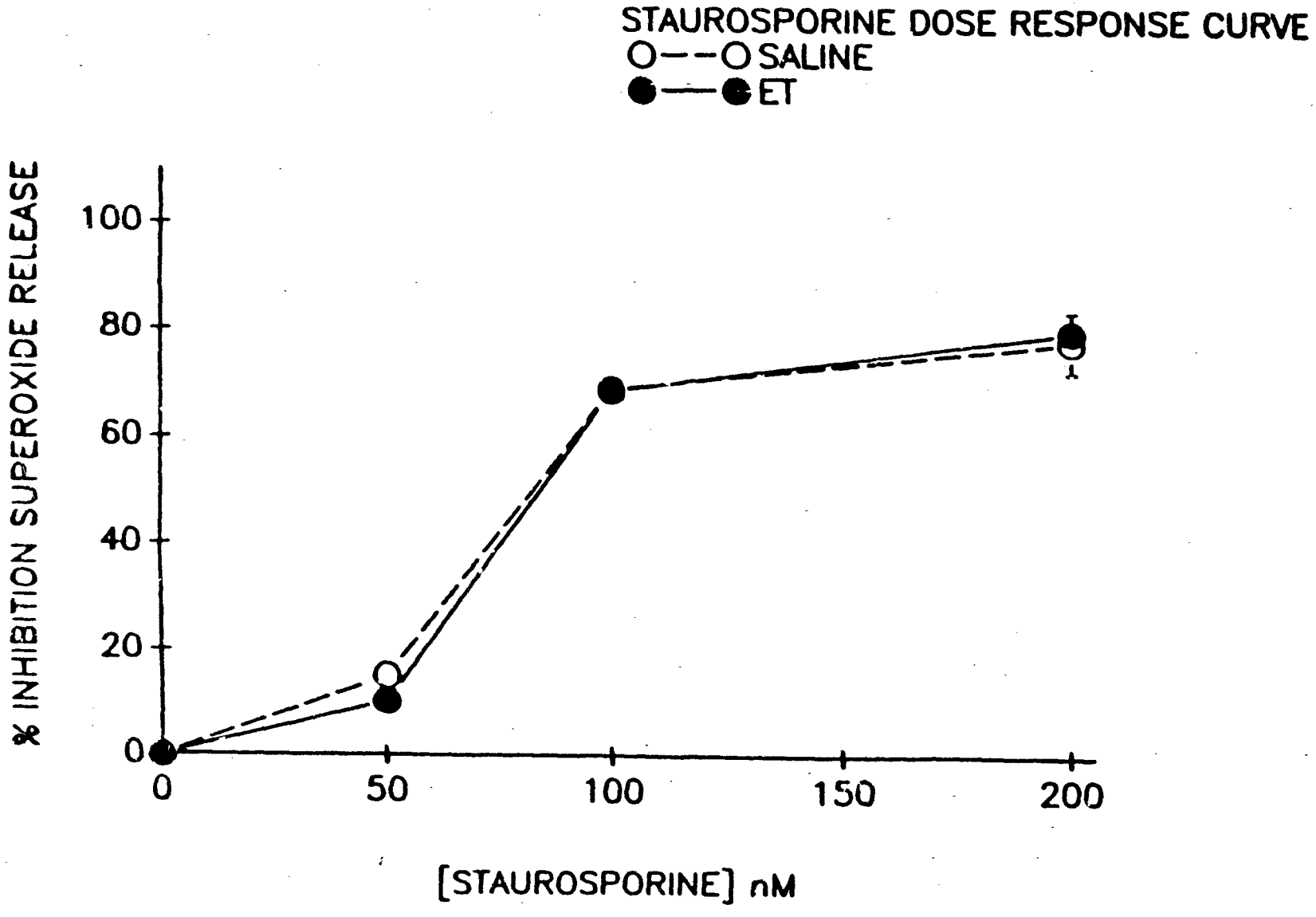

Fig. 4. Effect of staurosporine on SO release in AMs stimulated with OPZ (3.4 mg/10^6 cells). AMs were pre-incubated with the indicated concentration of staurosporine for 10 minutes at 37°C in a shaking water bath prior to beginning the SO assay.

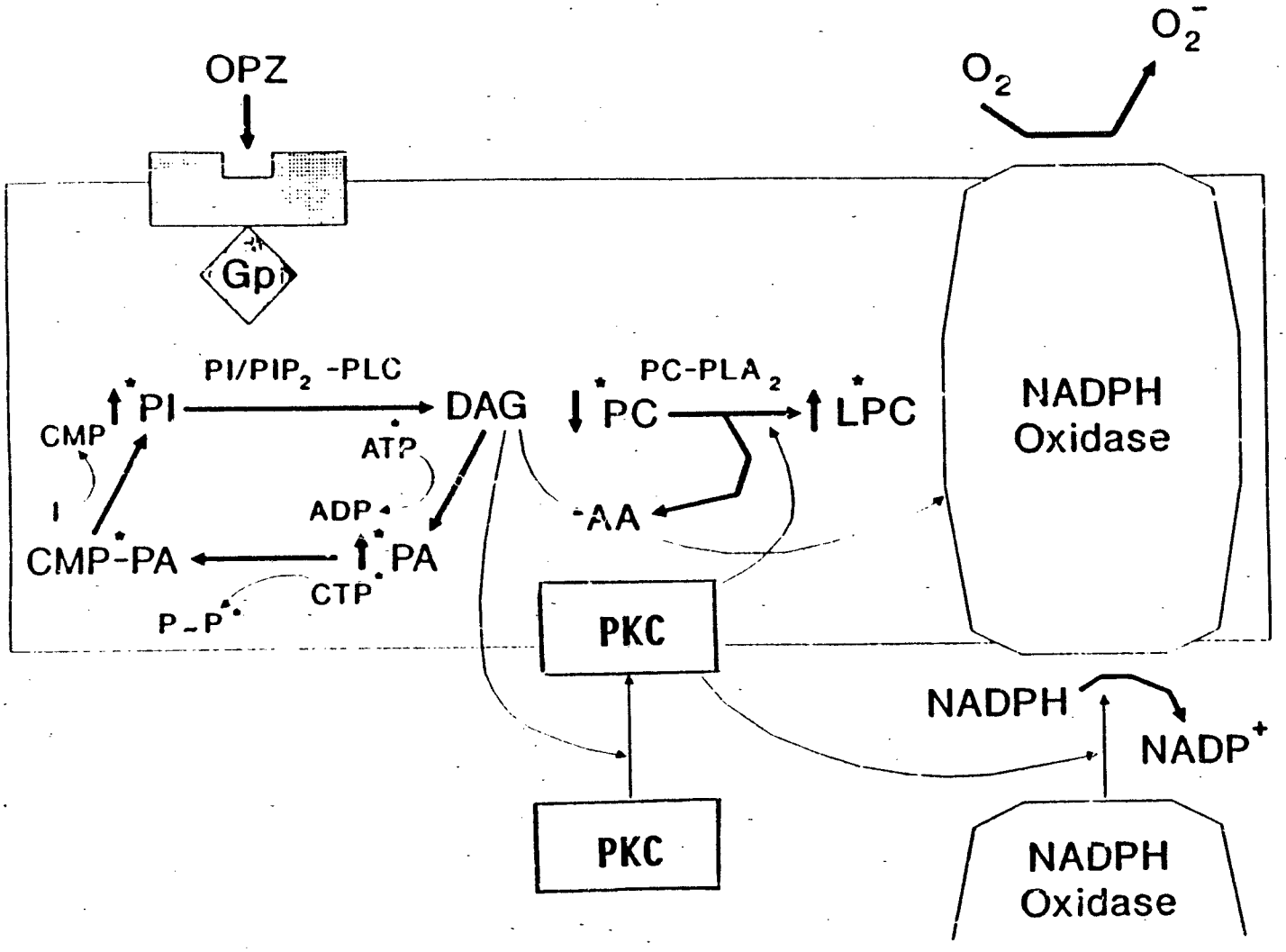

Fig. 5. Relationships of OPZ-stimulated signal transduction pathways (involving PLC-mediated stimulation of the PI cycle, PLA_2-mediated hydrolysis of PC, and PKC activation) to NADPH oxidase activation and O_2^- release.

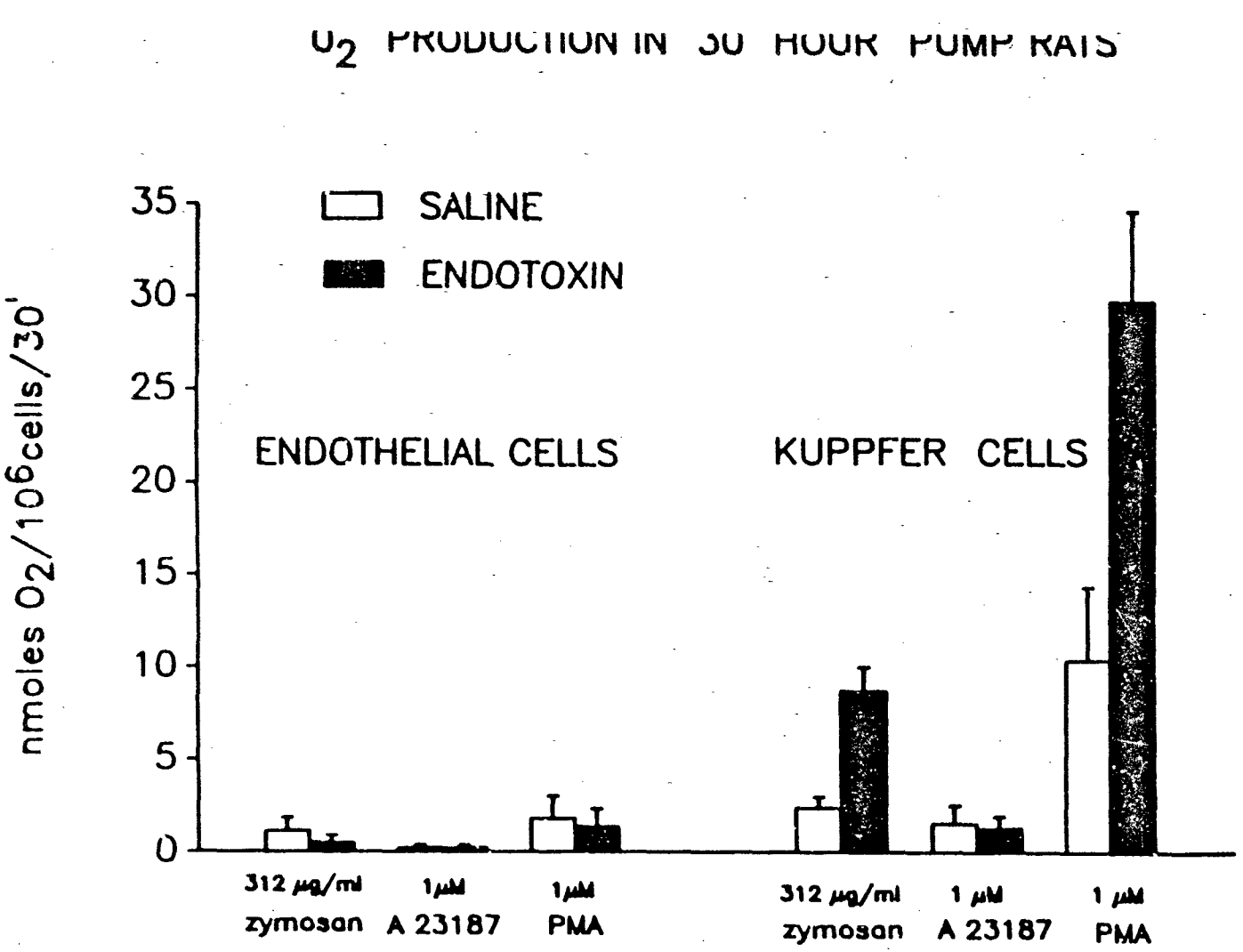

Fig. 6. Agonist-stimulated SO production by Kupffer and endothelial cells of rats continuously infused for 30 h with a nonlethal dose of E. coli ET or sterile saline via a surgically implanted osmotic pump. (n = 4)

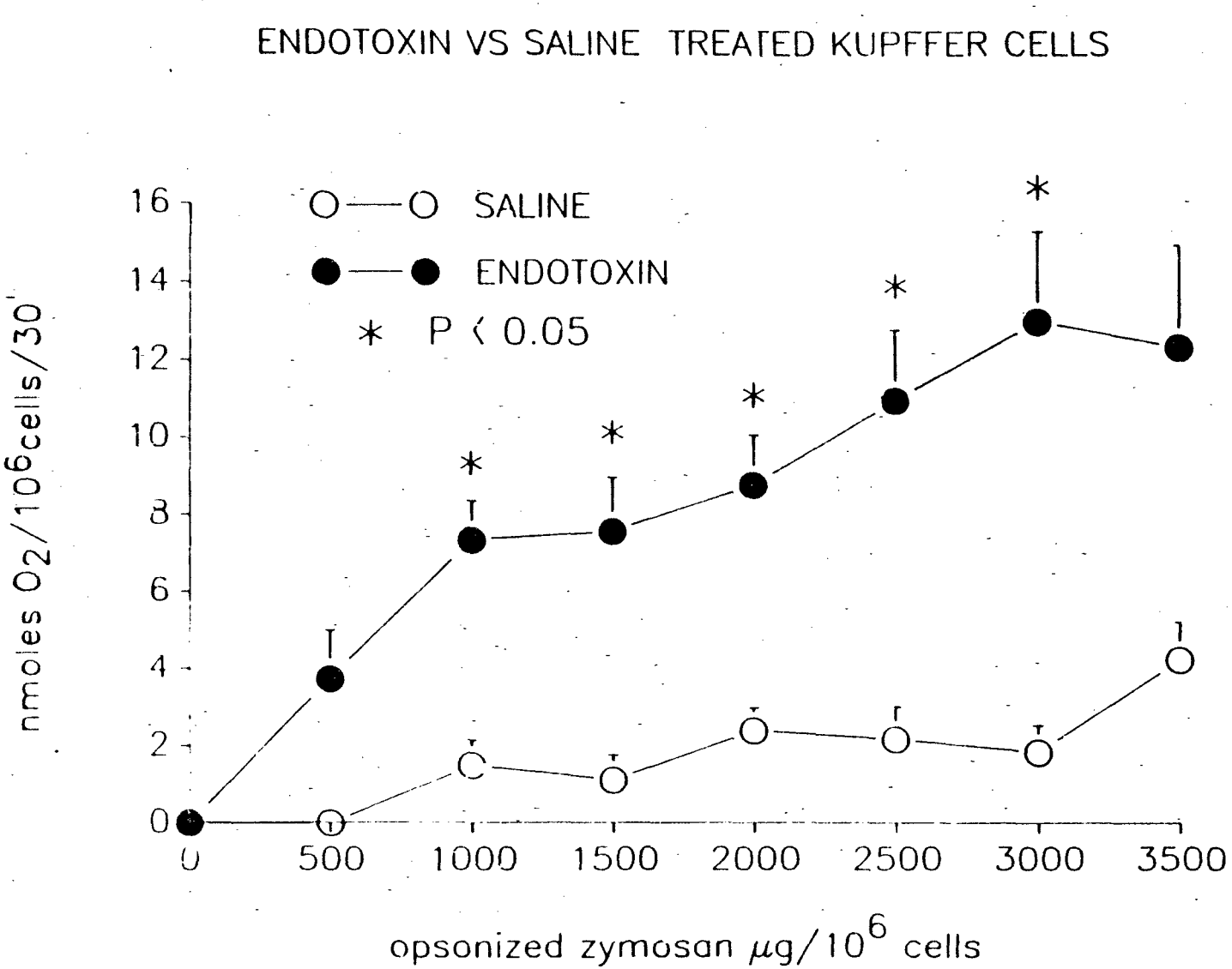

Fig. 7. **O2-dose response of SO release by Kupffer cells of rats infused continuously for 30 h with ET or sterile saline. (n = 4)**

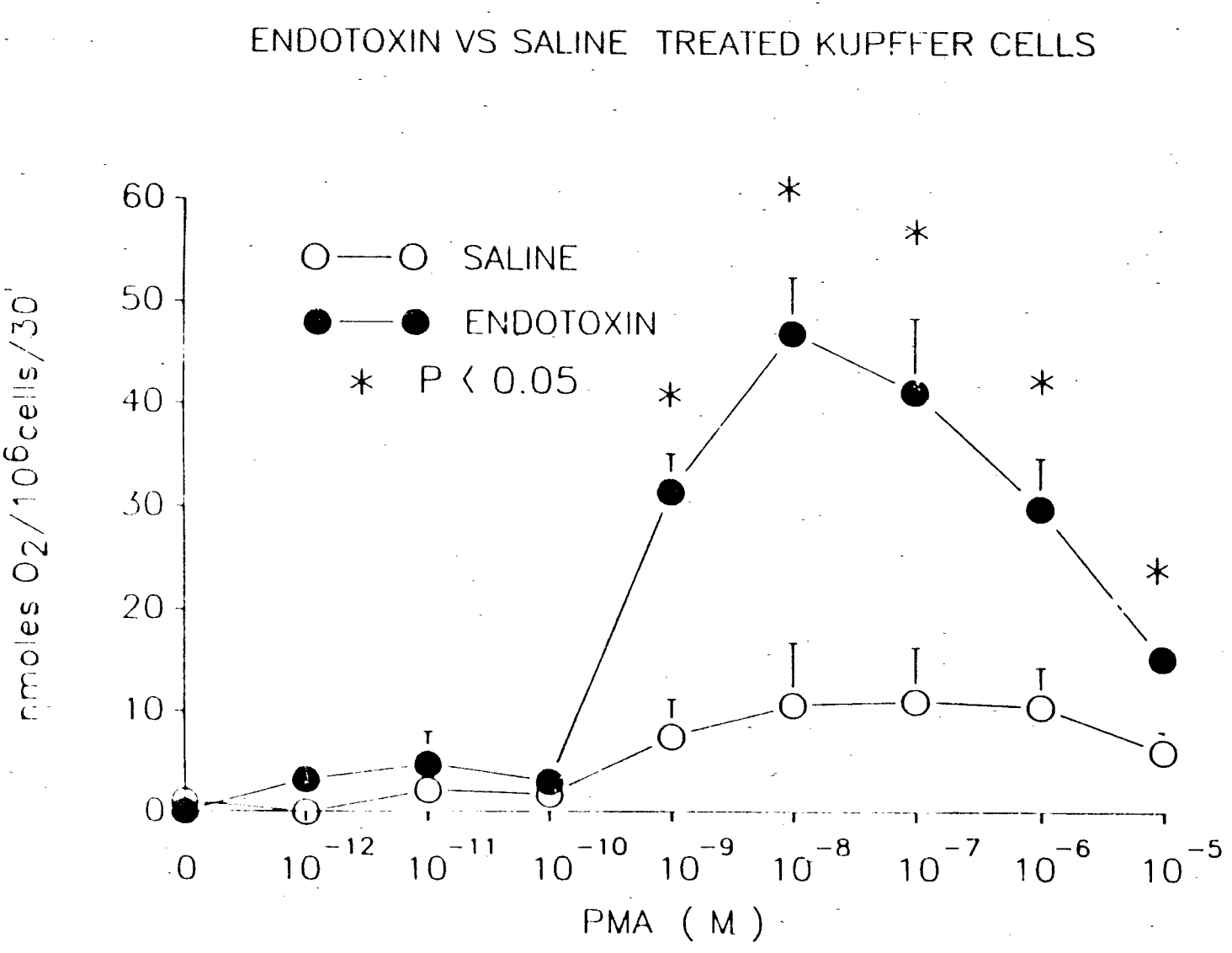

Fig. 8. PMA-dose response of SO release by Kupffer cells of rats infused continuously for 30 h with ET or sterile saline. (n = 4)